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(54) Title: HIGH THROUGHPUT SIZE-EXCLUSIVE METHOD OF SCREENING COMPLEX BIOLOGICAL MATERIALS FOR AFFINITY LIGANDS (57) Abstract <p>The invention encompasses an improved, rapid, size-exclusive method for screening complex biological materials, e.g., combinatorial libraries, natural products or samples, or mixtures of pure compounds, for small molecular weight ligands that bind specifically to a protein target, using size-exclusion separation, ultrafiltration, and mass spectrometry.</p>		

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TITLE OF THE INVENTION

HIGH THROUGHPUT SIZE-EXCLUSIVE METHOD OF SCREENING
COMPLEX BIOLOGICAL MATERIALS FOR AFFINITY LIGANDS

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CROSS REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional
Application 60/119,966, filed February 12, 1999, the
whole of which is herein incorporated by reference.

10

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR
DEVELOPMENT

Not applicable.

15

BACKGROUND OF THE INVENTION

The present invention relates to screening complex
biological materials, such as natural products and
combinatorial libraries, for affinity ligands, using
size-exclusion separation, ultrafiltration, and mass
spectrometry.

20

Testing complex biological samples for new drug
candidates in high throughput screening programs is a
successful strategy employed by the pharmaceutical
industry. Once an active sample is identified, however,
it can be difficult to isolate the active compound,
particularly from natural product extracts.

25

Natural extracts represent a highly chemically
diverse collection of compounds that make it very
difficult to isolate any single active compound. As most
successful drug compounds are of small molecular weight

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(less than 2,000 daltons), separations based on size can be a useful tool in assisting the isolation of active components.

Combinatorial chemistry offers the means to generate a large number of different chemicals simultaneously. Modern analytical methods allow the screening of such combinatorial libraries to select those compounds possessing desirable properties. However, these methods have their limitations, so that a need remains for a successful analytical methodology that provides high throughput screening of combinatorial libraries against biological targets for identification of active ligands.

Screening a library generally involves a binding or a functional assay to determine the extent of ligand-receptor interaction. Often, either the ligand or the receptor is immobilized on a solid surface (e.g. polymer bead or plate) and, after detection of the binding or the functional activity, the ligand is released and identified by a different means, for example, by mass spectrometry. Solid-phase screening assays offer faster isolation and identification of active analytes compared to the solution-based methods. On the other hand, the shift of combinatorial research to the creation of soluble non-peptide libraries, and limitations associated with heterogeneous assays, create a demand for a breakthrough technology for rapid and efficient screening of combinatorial libraries in solution. Solution-phase assays are desirable to increase screening specificity, but current methodologies involve iterative processes that are long and laborious.

Recently, electrospray ionization mass spectrometry (ESI-MS) has also been used in screening, directly or in conjunction with a solution-based screening method. The direct screening of combinatorial libraries by ESI-MS
5 relies on its ability to characterize non-covalent complexes of proteins bound to ligands (i.e., to distinguish between a protein target and its ligand, even a small one). Alternatively, mass spectrometry can be coupled, on-line or off-line, with solution-based
10 screening methodologies, as a final dimension for structure determination of biologically active compounds.

One method, the Hummel-Dreyer method, recently used for binding studies of small molecules to proteins, is based on size separation of the receptor protein and its
15 ligand by gel filtration. The approach of separating a receptor (i.e., a target protein) and a receptor-ligand complex from other small, unbound molecules on the basis of size differences, was first applied to the separation of antibody-peptide complexes from the rest of a peptide
20 library, with subsequent analysis of the ligands on reversed-phase high-performance liquid chromatography (RP-HPLC) (R.N. Zuckerman et al., *Proc. Nat. Acad. Sci., USA* 89:4505-4509 (1992), J.M. Kerr et al., *Bioorg. Med. Chem. Lett.* 3:463-468 (1993)).

25 Size-exclusion separation has also been applied to small molecule combinatorial libraries. Some work has been done on developing a methodology for selection and identification of ligands with high affinity to a biological target using a size-exclusion-complex
30 isolation procedure and mass spectrometry: e.g., Y. Dunayevskiy et al., *Rapid Comm. Mass Spec.* 11:1178-84

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(1997); M.M. Siegel et al., *J. Mass Spec.* 33:264-273 (1998);. These attempts to screen combinatorial libraries and other complex mixtures of compounds on the basis of size-exclusion separation have had limited success in actual application, particularly under the rigors of high throughput screening. One major problem has been the tendency of transfer lines and other conduits used in high-throughput, size-exclusion-complex isolation screening protocols, particularly transfer lines to reverse phase HPLC column, to become clogged and impassable after only 1-2 hours of operation. Such clogging, which occurs due to irreversible collection of the protein on the stationary phase of the HPLC column, necessitates frequent changes of the transfer lines. It also is a limiting factor in the overall effectiveness and on-line automation of such screening protocols. This effect is even more pronounced when dealing with complex biological mixtures such as natural samples that contain large MW biomolecules. Therefore, a need has remained for a size-exclusive screening method that can withstand high throughput conditions.

BRIEF SUMMARY OF THE INVENTION

The present method combines affinity interactions with size exclusion methods to enable rapid isolation and characterization of small molecule compounds from highly complex mixtures such as natural samples.

Also, the advantage of the present method's combination of techniques is that it allows one to screen pools of compounds simultaneously, instead of one compound at a time. The present invention provides an

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improved method of rapidly screening complex biological materials for affinity ligands using a unique combination of both size-exclusion separation (e.g., by gel filtration) and ultrafiltration steps, as well as mass spectrometry analysis. One advantage of the present method is that allows sustained, high-throughput screening, without having to replace HPLC columns for at least 7 days.

In general, the rapid screening method of the invention comprises the following main steps in the order given:

(i) mixing and incubating a protein target (TG) with a biological sample potentially containing a small molecular weight, affinity ligand (L), to form a reaction mixture, under conditions conducive to ligand/target (L/TG) complex formation in solution;

(ii) removing from the reaction mixture, unbound, small molecular weight material that is present in the biological sample, by using a size-exclusion medium that, based on molecular weight differences, separates out and retains small molecule compounds while allowing proteins or bound ligand/protein target (L/TG) complexes to pass through;

(iii) subjecting the size-excluded reaction mixture, now containing only large molecular weight materials like unbound proteins and bound L/TG complexes, to conditions conducive to L/TG complex dissociation, to yield free, small molecular weight ligand (L) and target (TG) in solution, and using a second size-exclusion medium, e.g., an ultrafiltration membrane, to isolate the free ligand from the protein target and other large molecules

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remaining in the size-excluded reaction mixture from step (ii); and

(iv) subjecting the isolated ligand to mass spectrometry (MS) in order to identify it structurally.

5

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

Figure 1 is a flowchart of the general steps of the method;

10 Figures 2A, 2B, and 2C show a schematic representation of an apparatus set-up for practicing an on-line embodiment of the screening method of the invention, at different timepoints in the method; and

15 Figures 3A-B show liquid chromatography-mass spectrometry (LC-MS) chromatograms from using an off-line embodiment of the method to screen for a ligand, acetazolamide, which binds to human carbonic anhydrase II.

DETAILED DESCRIPTION OF THE INVENTION

20 The invention provides an improved, high-throughput, size-exclusive method of screening complex biological material, using size-exclusion separation, ultrafiltration, and mass spectrometry. It allows the screening of several pools of compounds at one time, as
25 well as complex mixtures and natural samples containing molecules of very different MW sizes.

This invention uses a unique combination of steps: allowing binding of a small molecular weight, affinity ligand (L) to a protein target (TG) in solution; size-
30 exclusion separation of the ligand/protein (L/TG) complex from unbound, target-inactive material; subsequent

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dissociation of the L/TG complex and isolation of the target-binding ligand through another size-based separation (ultrafiltration) of the small ligand from large molecules (i.e., the target and other proteins);
5 and ligand identification using mass spectrometry (MS) alone or in conjunction with liquid chromatography (LC-MS).

"Small molecules" or "small molecular weight molecules" are understood in the art to refer to compounds with a molecular weight of about 2,000 Daltons or less. Advantageously, the method can detect and isolate from a screened complex biological sample, target-binding ligands of about 2,000 Daltons or less, more preferably 1000 Daltons or less. Examples of small
15 molecular weight ligands that may be identified by the present method include but are not limited to compounds within such complex biological materials as: combinatorial libraries (e.g., of peptides and the like); natural products, samples, or extracts; and mixtures of
20 pure compounds.

Large molecular weight compounds are generally those having a molecular weight of about 8,000, more typically 10,000 Daltons or higher. Examples include proteins as well as complexes of proteins noncovalently bound to small
25 molecule ligands.

The general method of the invention is outlined in the flowchart of Figure 1.

In one aspect, the invention provides a method of screening a complex biological sample for an affinity
30 ligand that binds to a protein target, comprising, preferably in the order given:

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(1) mixing a protein target and a complex biological sample in solution to form a reaction mixture;

5 (2) incubating the reaction mixture under conditions allowing complex formation by the target and any target-binding ligand present in the sample;

10 (3) passing the reaction mixture through a first size-exclusion medium that removes from the reaction mixture any small molecular weight compound having a molecular weight less than a first preset value (e.g., the first preset value may lie in the range of 5000 Daltons or less, with the cut-off being about 3000 Da)

15 (4) subjecting the size-excluded reaction mixture from step (3) to conditions promoting dissociation of any ligand/target complex into free ligand and free target;

20 (5) passing the reaction mixture resulting from step (4) through a second size exclusion medium that removes from the reaction mixture any molecule larger than a second preset value; and

25 (6) subjecting the reaction mixture resulting from step (5), to one of the following analyses: (a) mass spectrometry analysis or (b) liquid chromatography coupled on-line with mass spectrometry, thereby characterizing any small molecular weight ligand remaining in the reaction mixture resulting from step (5).

30 In another aspect, the method of the invention further comprises comparing the analytical results of

step (6) with a reference standard. The reference standard preferably comprises the analytical results (MS or LC-MS results) of subjecting either a sample of the protein target alone or a mixture of the protein target with a non-target-binding complex biological material sample, to steps(2)-(6) of the method.

The rapid screening method of the invention can be performed in an on-line or off-line format. Additionally, the method can further include a competitive-binding embodiment, as a control to determine whether ligand detected by the method binds to the selected protein target specifically (at the same site as a known competitive ligand) or non-specifically (at another site, e.g., by hydrophobic interactions).

Off-line Embodiment

The target protein TG is initially incubated with a biological mixture in solution for a time period sufficient to reach equilibrium or near equilibrium binding of any ligand to LC/MS or the target (e.g., about 5-60 minutes, preferably 5-10 minutes). Then the physical separation of an L/TG complex from unbound small molecule compounds is achieved on a size-exclusion medium, such as a gel filtration column (e.g., Pharmacia HR 10/10 columns), which provides separation of large molecules (e.g, target protein (TG) and other proteins in the sample) and large complexes (i.e., L/TG complex) from small molecular weight compounds. Non-binding small molecules in the target/sample mixture are retained by the gel-filtration column. Large molecules elute with the void column volume. Therefore, after this separation

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step, the size-excluded reaction mixture contains unbound TG and L/TG complex, as well as other large molecules initially present in the biological sample, all of which eluted in the void column volume.

5 Then a solution, comprising at least one organic solvent and at least one organic acid (e.g., 100 μ L acetonitrile ACN solution containing 0.1% trifluoroacetic acid (TFA)), is added to the size-excluded reaction mixture eluted from the gel filtration column. This step
10 creates conditions for protein denaturation and consequent release of bound ligand from target, i.e., dissociation of the bound L/TG complex. The whole resulting mixture is then loaded onto a second size-separation medium, such as an ultrafiltration membrane
15 having a small molecular weight cut-off, e.g., in the range of about 1,000-5000 Daltons (Da), preferably about 2,000-4,000, most preferably about 3,000 Da. Only molecules smaller than the ultrafiltration membrane's cut-off can pass through. Thus, this second size-
20 separation step provides isolation of the dissociated ligand molecules from all large molecules in the screened biological sample. Small molecules remaining in the mixture after the first size-exclusion step (i.e., an affinity ligand having the desired affinity to the
25 protein target), pass through the ultrafiltration membrane, while the membrane surface retains all large molecules (i.e., target and other large MW molecules originating from the sample).

30 The remaining, ultrafiltered reaction mixture or material is subjected to MS alone or by liquid chromatography coupled on-line with MS (LC-MS), to

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analyze any target-binding ligand isolated by the ultrafiltration step.

On-line Embodiment

5

In an on-line format, the size-exclusion column (SEC), the ultrafiltration membrane, and liquid chromatography-mass spectrometry (LC-MS) system are assembled together on-line. One of the possible
10 instrumental arrangements to carry out the method of the invention is shown in Figures 2A, 2B, and 2C, which depict the method and instruments at different time points. Figure 2A shows the isolation and trapping of large molecular weight biomolecules in the loop.
15 Figure 2A shows the transfer of the isolated large molecular weight biomolecules onto an ultrafiltration membrane, together with application of conditions conducive to ligand/target complex dissociation, the separation of any dissociated ligand from the target, and
20 deposition of the isolated ligand on a reverse-phase LC column. Fig. 2C shows the characterization of the ligand, after ultrafiltration, by LC-MS analysis.

In the on-line method, after incubation of the reaction mixture for a time sufficient to allow target-
25 ligand binding (e.g., within about 5-60 minutes), the mixture is injected into the SEC, and large molecules come out first, non-retained by the SEC stationary phase of the SEC. Small molecules are retained in the pores of the SEC stationary phase. The chromatographic peak
30 corresponding to large molecules is forwarded into a sample loop (Figure 2a), and the remaining flow from the

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SEC is then diverted into waste. The solution containing organic solvent (e.g., acetonitrile (ACN)) acid (TFA) and organic acid (e.g., acetic acid or trifluoroacetic acid (TFA)) is then added to the sample in the loop, in order to provide conditions for L/TG complex dissociation. At the same time, sample is transferred into the chamber of the ultrafiltration membrane (Figure 2b). Then the sample is pumped through the ultrafiltration membrane directly into an MS or into an LC column for further LC-MS analysis of the released ligand (Figure 2c). Only the released small molecule ligand is able to pass through the membrane, and is subsequently identified by MS or LC-MS.

15 Competitive Binding Embodiment

Another embodiment of the invention combines the use of competitive binding along with the described screening assay. Utilization of a known competitive ligand (CL) that binds to a selected target allows determination of whether the active ligand extracted from a biological mixture is bound to a specific binding site or known site of the target protein. In this case, a CL that binds to a known S.H. is added to the reaction mixture of target and biological sample containing a small molecular weight affinity ligand at the first, mixing and incubation stage, and during mass spectrometry, both L and CL signals are monitored. If the MS signal of the extracted ligand L is unaffected by the presence of CL in the binding mixture, one concludes that the identified ligand L binds to a different site from the CL binding site of TG. This competitive-binding approach thus allows one to

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identify ligands binding to different sites of the target. For example, this could be used to quickly eliminate non-specific binders detected during the screening method, as candidate compounds for regulating the activity of the target protein. Instead, one can focus further screening, testing and/or characterization efforts on those ligand molecules (L) whose binding to the target diminishes upon inclusion of the known competitive ligand in the reaction mixture. Such a result suggests that the candidate ligand from the screened sample thus binds to the same site on the protein target as does the known competitive ligand.

In the competitive binding embodiment of the method, the reference standard for determining whether a screened sample contains a specific target-binding ligand, comprises the MS or LC-MS results of subjecting a mixture of the protein target and the known competitive ligand, in the absence of any other target-binding ligand, to steps(2)-(6).

Exemplary Protocols and Materials

Exemplary Gel Filtration Isolation Protocol

Stock solutions of candidate compounds used for assaying their binding to a protein target (e.g., human carbonic anhydrase II (CAII)), can be prepared by dissolving them in a 1:1 (v/v) mixture of acetonitrile (HPLC Grade; Fisher Scientific, Springfield, NJ, USA) (ACN) and buffer A, and diluting to a concentration of 10 times higher than the desired concentration in the binding assay. The target protein stock solution can be

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prepared by dissolving and diluting the target in buffer A. Binding mixtures consisted of 50 μ L target stock with 5 μ L of the candidate compounds stock. The reaction mixtures are incubated at room temperature for 1 hr, after gentle mixing by pipette. The gel filtration columns (e.g., Sephadex G25 fast-desalting spin columns or Pharmacia HR 10/10 columns, in an off-line format of the method) are handled according to the manufacturer's instructions. For instance, G25 columns are first washed with 100 μ L water and centrifuged at 3000 rpm (~900 g) at 4°C for 5 min. The reaction mixtures are then loaded on the columns and centrifuged as before. Unbound compounds should be retained on the columns, while any protein, bound or unbound to low molecular weight candidate compounds, should flow through the column. Protein complexes in the flow-through of spin columns are collected. The protein-ligand complex in the eluted fraction is then denatured by adding 100 μ L ACN solution containing 0.1% trifluoroacetic acid (TFA) (Sigma Chemical CO.), incubating for 5 min at room temperature, and vortexing for 15 seconds. The denatured protein is removed by centrifugation at 10 000 g for about 30 seconds.

25 Exemplary Ultrafiltration Protocol

The supernatant liquid resulting from the gel filtration protocol is then applied to the second size-exclusion separation medium, preferably an ultrafiltration membrane. Examples are ultrafiltration membranes having a 20,000, 10,000, or 3,000 Dalton cut-off sold as Microcon-3, by Amicon, Beverly, MA, USA.

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Preferred are membranes with a cut-off of about 10,000 Da (which lets pass about 1% of molecules over 30,000 Da), or less. A membrane with a 3,000 Da cut-off lets pass only about 0.1% of materials over 30,000 Da. Most preferred are ultrafiltration membranes that can withstand 100% organic solvents (e.g., ACN) and organic acids (e.g., TFA or acetic acid).

Ultrafiltration membranes having other, small molecular weight cut-offs, e.g., within the range of about 1000-5000 Da, preferably 2000-4000 Da, are also available commercially (e.g., All Filtron Co., Northborough, MA 5000 Da cut-off).

Exemplary MS or LC-MS Protocol

The ultrafiltered liquid (containing any candidate ligand that bound to the target), is then analyzed by mass spectrometry alone or by liquid chromatography coupled to mass spectrometry, for the presence of released small molecular weight compounds, after lyophilization and re-suspension in small volumes.

Mass spectrometry: Analysis can be performed on, e.g., ion trap triple quadrupole mass spectrometers LCQ (Thermo-Quert Corporation, San Jose, CA, USA) or APCI. The electrospray voltage is generally maintained in the range of about +4.5-4.75 kV. Ion optics settings are optimized on the day of the analysis to provide the maximum efficiency of ion to the detector. The effective mass range is generally from m/z 150 to m/z 700 at a rate of about 1 s/scan.

Liquid chromatography: For example, samples can be introduced through a HP1100 (Hewlett Packard Paulo Alto,

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CA USA) chromatography operating in the gradient mode at a flow rate of 1 ml/min. An Inertsil C18 base-deactivated microbore column (4.6 mm x 10 cm) from MetaChem Technologies (Torrance, CA, USA) is used for sample separation. The mobile phase gradient is Milli-Q H₂O + ACN 90/10 (v/v) to a H₂O + ACN 0/100 (v/v) in 15 minutes. Samples are introduced through a Rheodyne Model 7125 injector (Cotati, CA, USA) with a 10 µL external loop. The sample injection volumes are generally 1-19µL.

10

Example I (Off-line format)

The off-line screening procedure of the invention is demonstrated using human carbonic anhydrase II (CAII) as a target, and a natural sample (NS) inactive toward CAII (e.g., inert fungal extract) that is spiked with a known, small molecule ligand that binds specifically to the active site of CAII, acetazolamide (AZ). Approximately 20 µM of CAII was mixed and incubated with approximately 1 microgram of an inactive natural sample containing about 10 µM of AZ in a buffered solution such as phosphate buffered saline, pH 7.0, 1% DMSO, for final CAII and AZ concentrations of about 1 micromolar. Size-exclusion chromatography was performed using a Pharmacia HR 10/10 column at 4 ml/min using a mobile phase of 200 mM ammonium acetate, pH 7.0. Typically, the excluded volume containing the target protein (CAII) with bound ligand (AZ), elutes from the column in about 0.6-0.7 minutes under such conditions.

Figure 3A shows an LC-MS chromatogram of a blank experiment, where about 20 µM CAII alone was passed through the entire method of the invention. Figure 3B

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depicts the LC-MS chromatogram from performing the same with that amount of CAII incubated with the inactive natural sample (NS) alone. There are no additional peaks detected, compared to the blank experiment, which indicates the absence of target-binding ligands from the inactive biological sample. Figure 3C [Orig. Fig. 1c] shows the LC-MS results from performing the same screening process with a natural (NS) sample now containing about 10 μ M of the CAII-binding ligand, acetazolamide (AZ). Active ligand was successfully extracted from the inactive complex biological matrix as a result of the screening method of the invention. As shown in Figures 3A-3C, target CAII (TG) peaks are a series of chromatographic peaks having retention times of 10.5 min. and longer. The ligand AZ peak has a retention time of 5.9 min.

It is understood that one of ordinary skill in the art will be able to effect minor variations to the embodiments of the methods herein described, without departing from the spirit and scope of the invention as set forth in the claims.

CLAIMS

What is claimed is:

1. A method of screening a complex biological sample
5 for an affinity ligand that binds to a protein target,
comprising:
 - (1) mixing a protein target and a complex
biological sample in solution to form a reaction
mixture;
 - 10 (2) incubating the reaction mixture under
conditions allowing complex formation by the target
and any target-binding ligand present in the sample;
 - (3) passing the reaction mixture through a
first size-exclusion medium that removes from the
15 reaction mixture any small molecular weight
compounds each having a molecular weight less than a
first preset value;
 - (4) subjecting the size-excluded reaction
mixture from step (3) to conditions promoting
20 dissociation of any ligand/target complex into free
ligand and free target;
 - (5) passing the reaction mixture resulting
from step (4) through a second size exclusion
medium that removes from the reaction mixture any
25 molecule larger than a second preset value; and
 - (6) subjecting the reaction mixture resulting
from step (5), to one of the following analyses:
(a) mass spectrometry analysis or (b) liquid
30 chromatography coupled on-line with mass
spectrometry analysis.

2. The method of claim 1, wherein the first size-exclusion medium removes molecules having a molecular weight of about 2,000 daltons or less.
- 5 3. The method of claim 1, wherein the first size-exclusion medium removes molecules having a molecular weight of about 1,000 or less.
- 10 4. The method of claim 1, wherein the first size-exclusion medium comprises a gel filtration or size exclusion HPLC column.
- 15 5. The method of claim 1, wherein step (4) comprises adding to the size-excluded mixture from step (3), a solution comprising an organic solvent and an organic acid.
- 20 6. The method of claim 1, 4, or 5, wherein the second size-exclusion medium comprises an ultrafiltration membrane.
- 25 7. The method of claim 1, 4, or 5, wherein the second size-exclusion medium removes from the reaction mixture, molecules having a molecular weight of about 10,000 daltons or more.
- 30 8. The method of claim 1, 4, or 5, wherein the second size-exclusion medium removes from the reaction mixture, molecules having a molecular weight of about 3000 daltons or more.

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9. The method of claim 1, 4, or 5, wherein the second size-exclusion medium removes from the reaction mixture, molecules having a molecular weight of about 2000 daltons or more.

5

10. The method of claim 6, wherein the ultrafiltration membrane removes from the reaction mixture, molecules having a molecular weight of about 10 000 daltons or more.

10

11. The method of claim 6, wherein the ultrafiltration membrane removes from the reaction mixture, molecules having a molecular weight of about 3000 daltons or more.

15

12. The method of claim 6, wherein the ultrafiltration membrane removes from the reaction mixture, molecules having a molecular weight of about 2000 daltons or more.

20

13. The method of claim 1, 4, or 5, further comprising:
(7) comparing the analytical results of step (6) with a reference standard.

25

14. The method of claim 13, wherein the reference standard comprises the analytical results of subjecting either a sample of the protein target alone or a mixture of the protein target with a non-target-binding complex biological material sample, to steps(2)-(6).

30

15. The method of claim 1, 4, or 5, further comprising, in step (1), including a known competitive ligand that

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binds to the target in the reaction mixture prior to step (2).

5 16. The method of claim 15, wherein the concentrations of the known competitive ligand and the target are approximately equimolar.

10 17. The method of claim 15, wherein the known competitive ligand concentration is within a range of approximately twice to 10 times the target concentration.

18. The method of claim 15, wherein the known competitive ligand concentration is approximately 5 times the target concentration.

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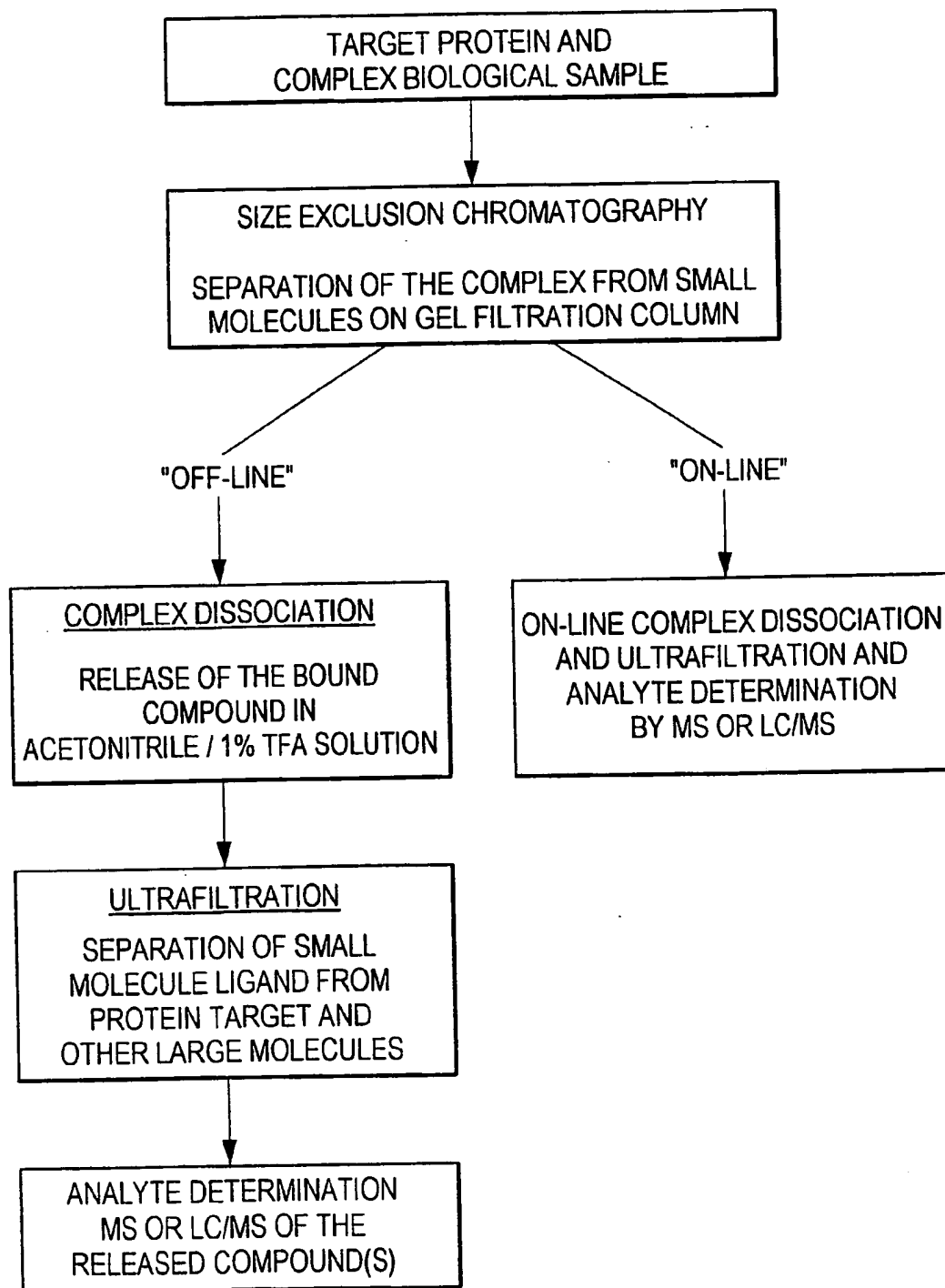
19. The method of claim 15, further comprising:

(7) comparing the analytical results of step (6) with a reference standard.

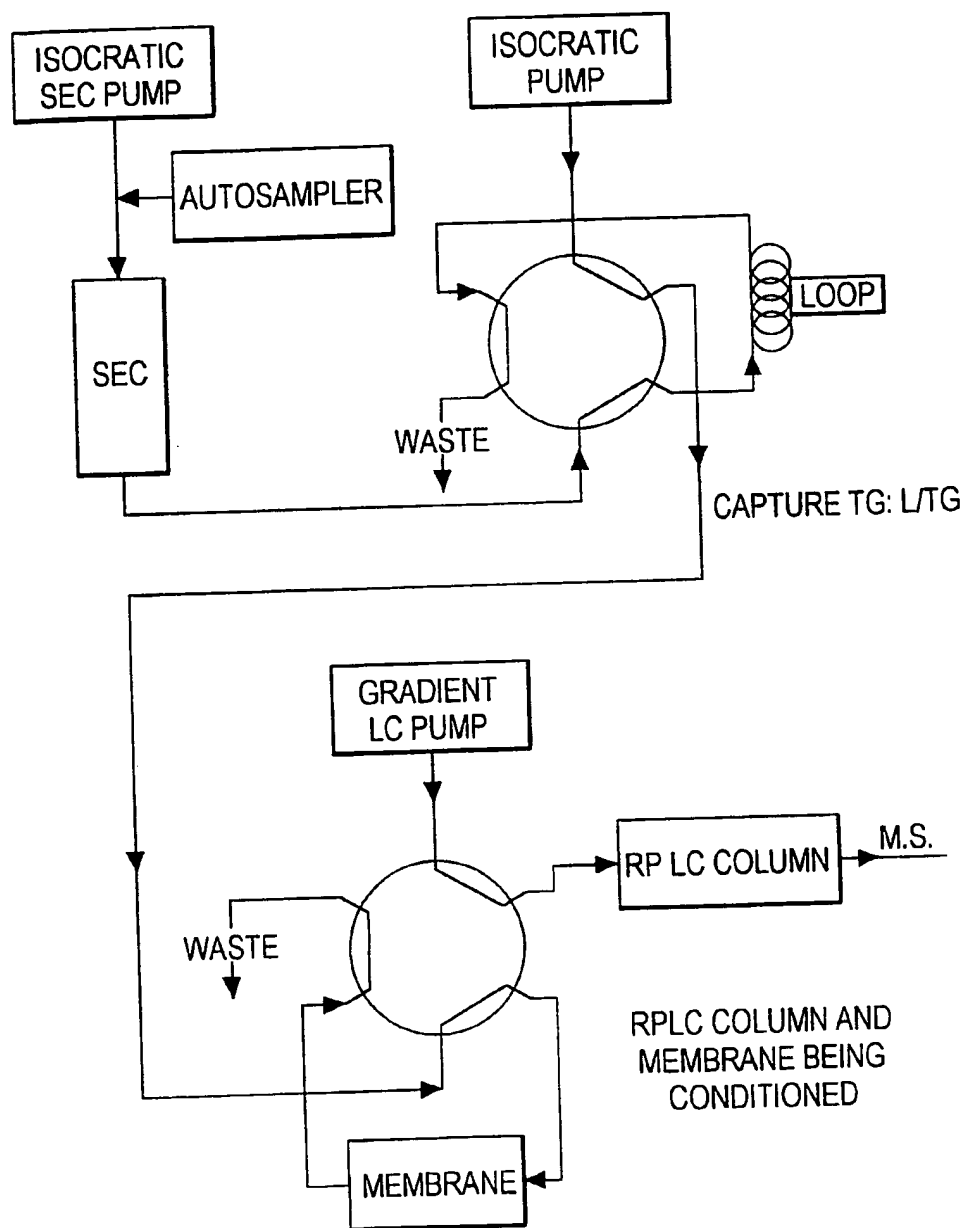
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20. The method of claim 19, wherein the reference standard comprises the analytical results of subjecting a mixture of the protein target and the known competitive ligand, in the absence of any other target-binding ligand, to steps (2)-(6).

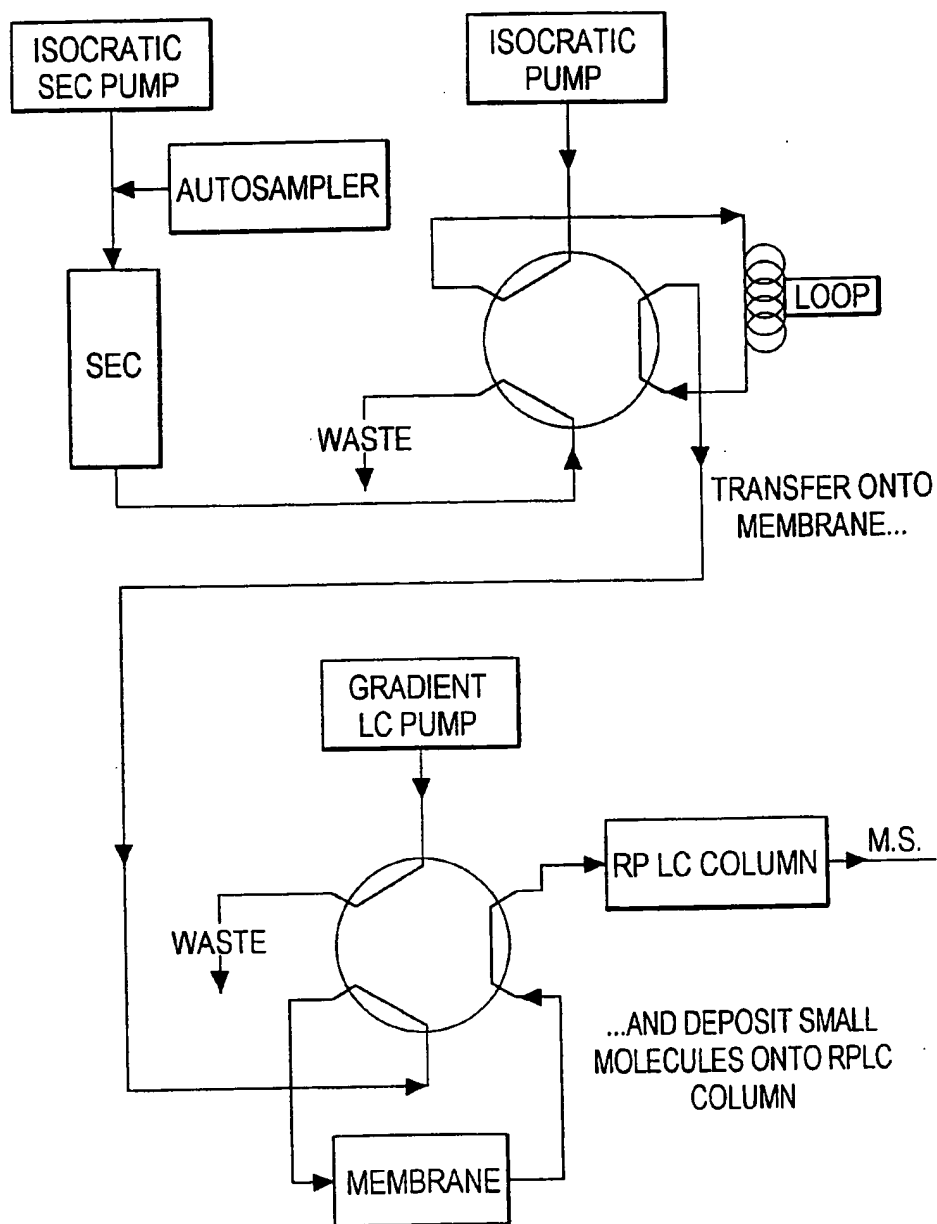
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**FIG. 1**

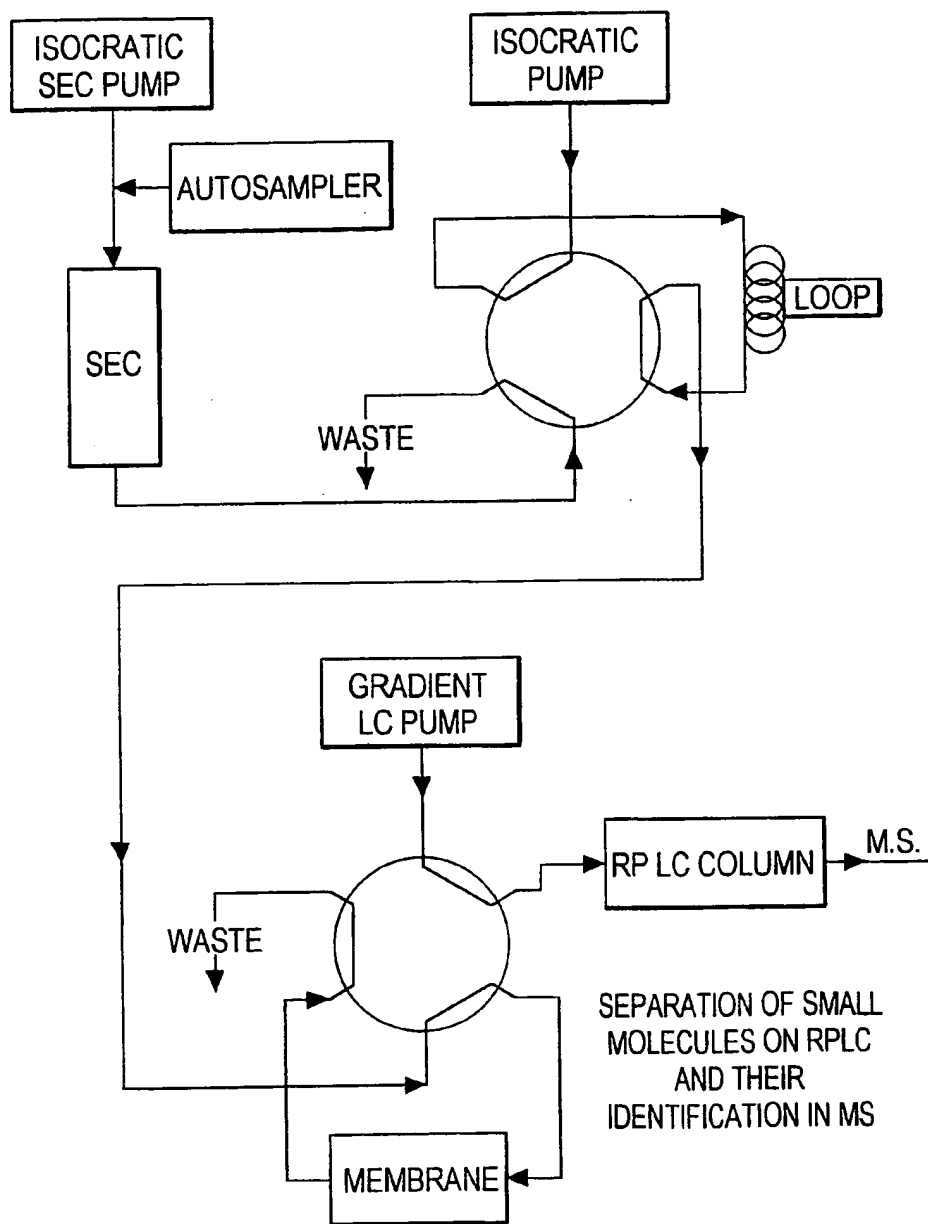
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**FIG. 2A**

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**FIG. 2B**

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**FIG. 2C**

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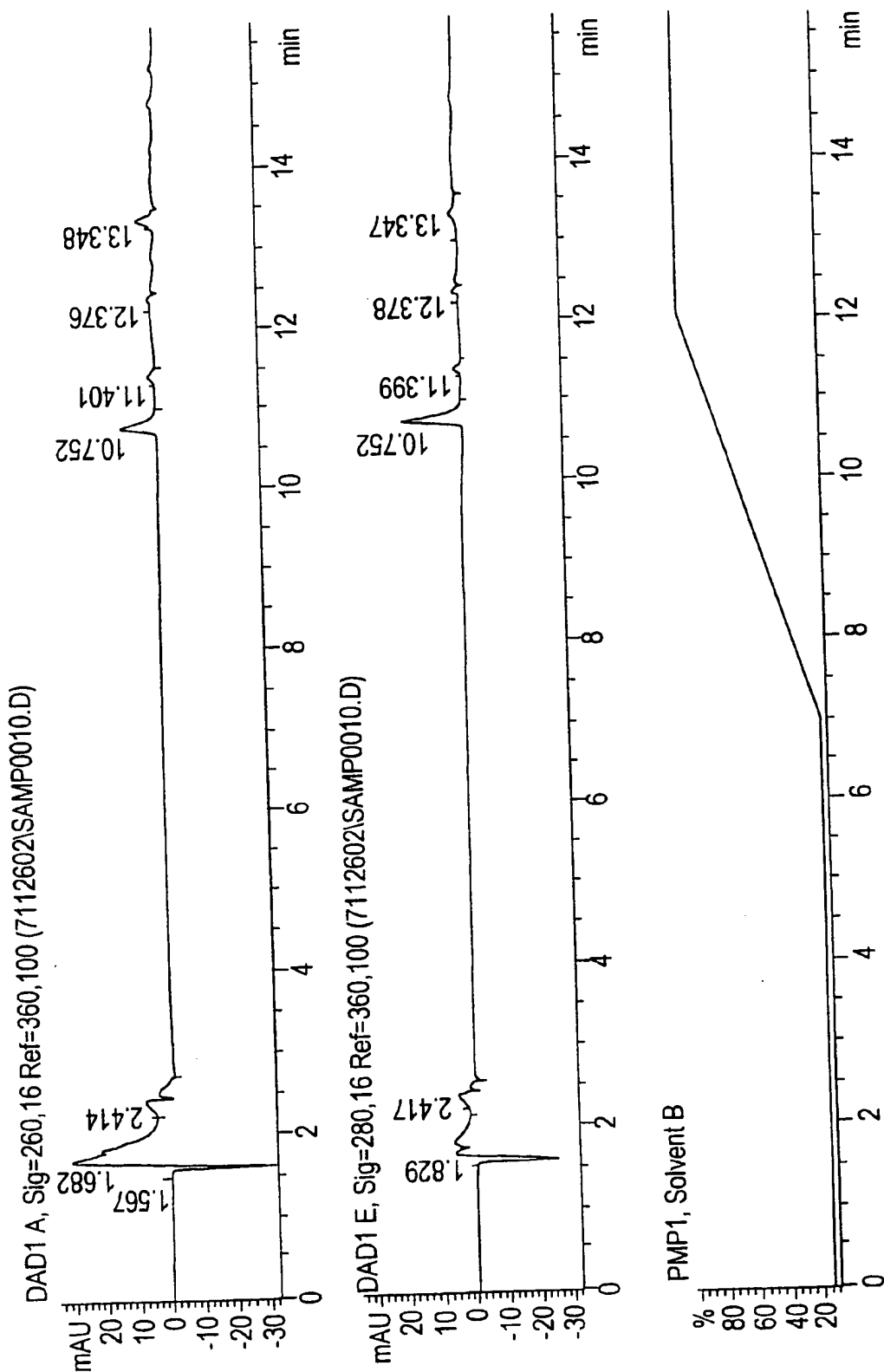


FIG. 3A

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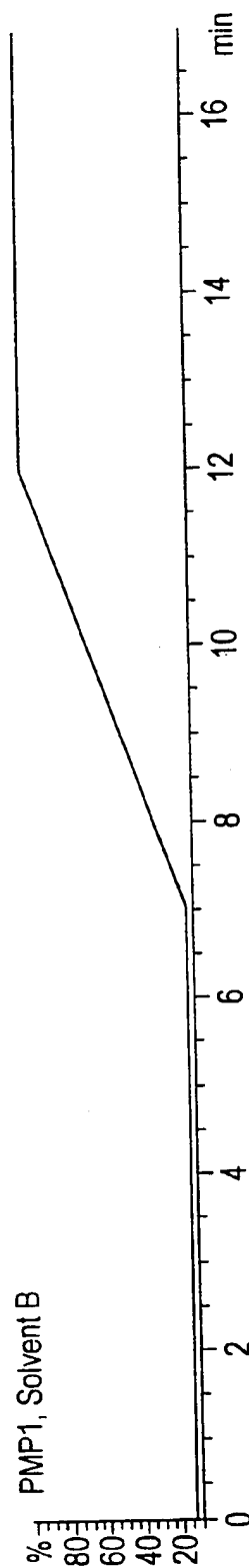
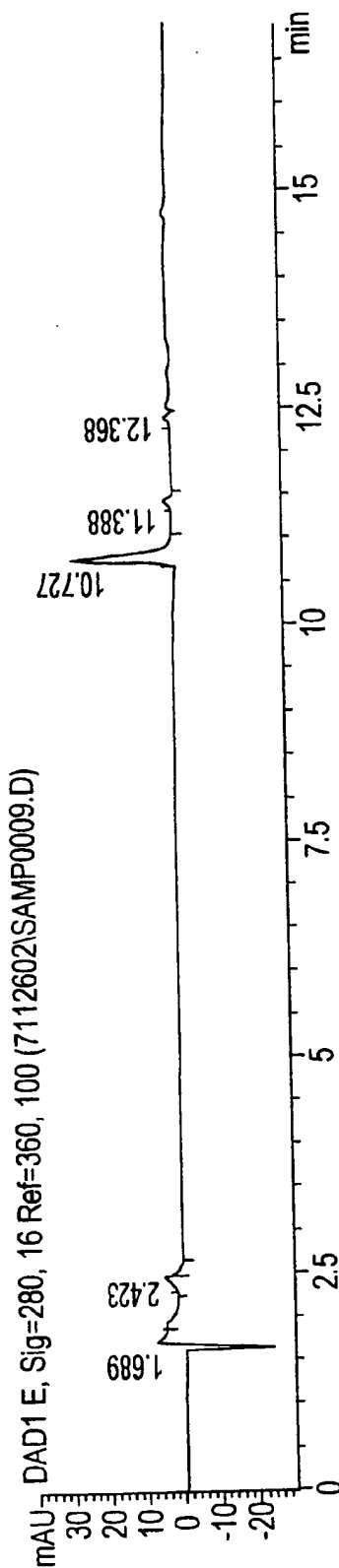
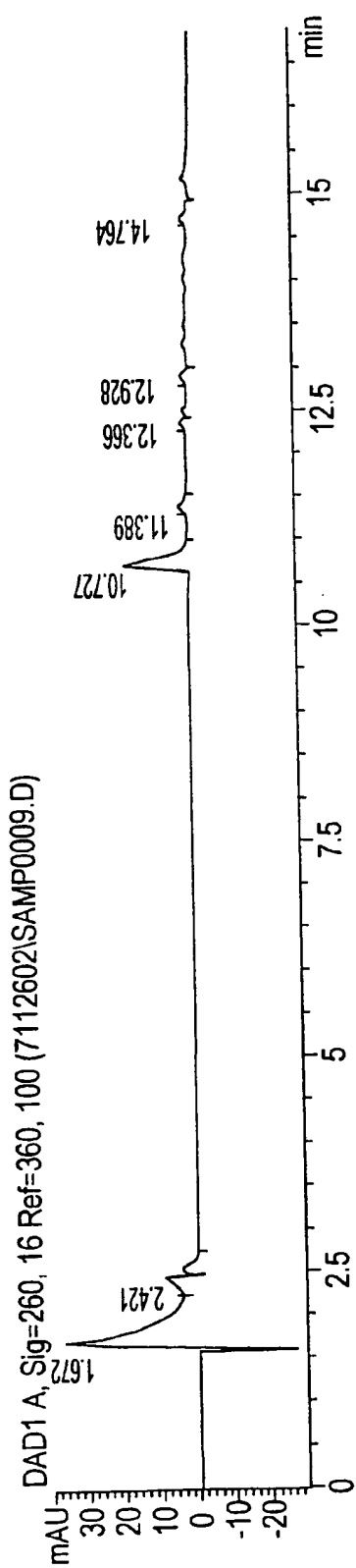


FIG. 3B

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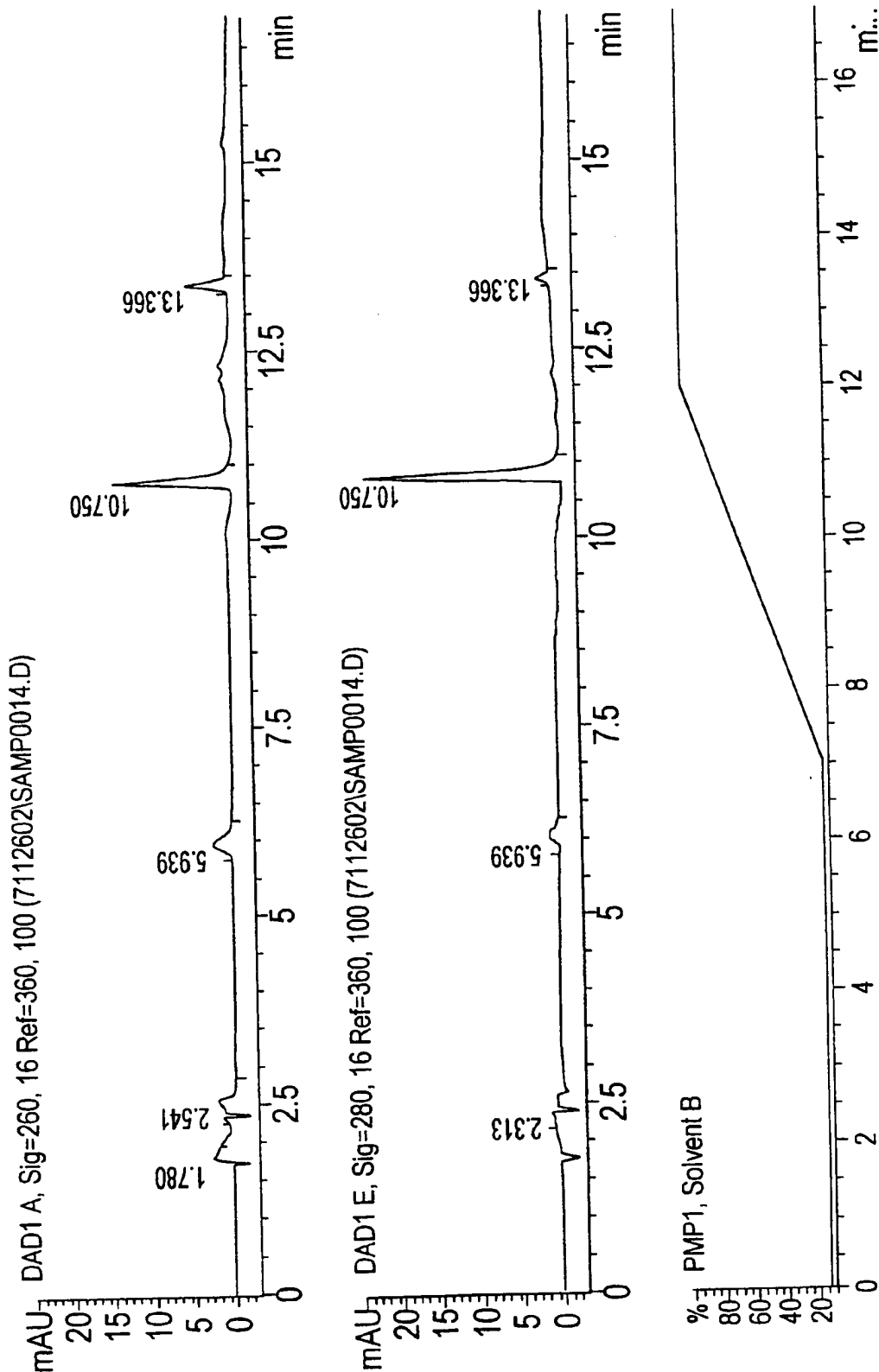


FIG. 3C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/03562

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/537

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/538; 436/528; 435/532; 436/533; 436/534; 436/86; 436/89; 436/518; 436/528

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, databases included: REGISTRY, CAPLUS, CAOLD, U.S. PATFULL; BIOSIS; MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database HCAPLUS on STN, American Chemical Society (Washington, DC), No. 1997:551555, DUNAYEVSKIY, YM. et al., 'Mass Spectrometric Identification Of Ligands Selected From Combinatorial Libraries Using Gel Filtration,' abstract, Rapid Commun. Mass. Spectrom., 1997, 11(11), 1178-1184, see entire document.	1-20
X	KAUR et al. Affinity Selection and Mass Spectrometry-Based Strategies to Identify Lead Compounds in Combinatorial Libraries. Journal of Protein Chemistry. 1997, Vol. 16, No. 5, pages 505-511, see entire document.	1-20
X,P	US 5,891,742 A (DOLLINGER ET AL) 06 APRIL 1999 (06/04/99), see entire document.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 APRIL 2000

Date of mailing of the international search report

06 JUN 2000

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/03562

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DUNAYEVSKIY et al. Simultaneous Measurement of Nineteen Binding Constants of Peptides to Vancomycin Using Affinity Capillary Electrophoresis-Mass Spectrometry. J. Med. Chem. 28 February 1998, Vol. 41, pages 1201-1204, see entire document.	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/03562

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

436/538; 436/528; 435/532; 436/533; 436/534; 436/86; 436/89; 436/518; 436/528